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Involvement of calmodulin-dependent phosphorylation in the activation of brainstem tryptophan hydroxylase induced by depolarization of slices or other treatments that raise intracellular free calcium levels

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Several lines of evidence now indicate that both crude supernatant and purified preparations of brainstem or midbrain tryptophan hydroxylase, the rate-limiting enzyme in serotonin synthesis [tryptophan-5-monooxygenase; L-tryptophan, tetrahydropterin:oxygen oxidoreductase (5-hydroxylating), EC 1.14. 16.4], can be activated by calcium–calmodulin-dependent protein kinase [1–3]. Tryptophan hydroxylase also becomes activated as a result of *in vivo* electrical stimulation [4] or drug-induced increases in firing of serotonergic neurons in the brain.* In both cases a phosphorylation reaction has been implicated in the activation process which is sensitive to frequency of stimulation and is readily reversible. Other studies on slices of brainstem revealed that potassium depolarization produces a calcium-dependent activation of the enzyme [5]. Indeed a variety of manipulations that raise intracellular levels of free calcium (A23187, metabolic inhibitors, methylxanthines) activate this enzyme [6–8]. A role for calmodulin, in addition to that of calcium, in mediating the activation of tryptophan hydroxylase is supported by the inhibitory effects of haloperidol and fluphenazine, antipsychotic drugs, on the activation process induced in the slices [7, 8]. The present experiments were designed to explore further the involvement of calmodulin-sensitive phosphorylation in the activation of tryptophan hydroxylase that results from treatments to brainstem slices.

Methods

The procedures used in this study have already been outlined in detail in previous reports [5, 6]. Brainstems (diencephalon, midbrain and pons-medulla) from male Sprague–Dawley rats were cut into 250 μm slices with a Dupont–Sorvall MT-2 tissue chopper, and each sliced brainstem was incubated at 25° in oxygenated medium (150 mM NaCl, 6 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 10 mM Tris acetate buffer, pH 7.4) to which various deletions, substitutions or additions were made as indicated in Results. The slice preparations were exposed to the treatment medium for 10 min. Calcium-free control medium containing 0.1 mM ethylene glycol bis (β-aminoethylether)-*N*,*N*′tetraacetic acid (EGTA) was used in experiments with 3-isobutyl-1-methylxanthine (IBMX) and guanidine. The drugs W-7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride] (Sigma Chemical Co., St Louis, MO) and polymixin B (United States Biochemical Corp., Cleveland, OH) were preincubated with the slices in control medium for 10 min in addition to being included in the incubation medium along with the calcium-mobilizing agents. Low speed supernatant extracts (30,000 *g* for 30 min) of tryptophan hydroxylase were prepared from brainstem slices that had been pelleted by brief centrifugation. The tissue was homogenized in 2 vol. of 0.05 M Tris-acetate buffer, pH 7.4, by means of a Tissumizer (45 sec at setting 50) (Tekmar, Cincinnati, OH) instead of a glass homogenizer. The supernatant fraction was made 2 mM with dithiothreitol, subjected to gel filtration, and assayed for tryptophan hydroxylase in the presence of 50 μM D,L-6-methyl-5,6,7,8-tetrahydropterin (6-MPH_4) and 200 μM L-tryptophan [5, 6]. All additions to the enzyme assay such as alkaline phosphatase or phosphorylating conditions are indicated in the Results. The 5-hydroxytryptophan (5-HTP) formed in the assay was isolated and quantitated by HPLC with electrochemical detection [4]. Enzyme activities are the mean ± SEM of at least four experiments and are expressed in pmoles 5-HTP formed per mg protein per min. The significance of differences in enzyme activity between the various treatment groups was determined by ANOVA and the Newman–Keuls test.

Results and discussion

Table 1 shows that the increases in tryptophan hydroxylase activity induced by the four different treatments to the slices (potassium depolarization, ionophore A23187, the methylxanthine, IBMX or the metabolic inhibitor, guanidine) were all reversed by incubation of the enzyme extract with alkaline phosphatase prior to addition of the assay reactants. In contrast, no change in activity of enzyme from control slices occurred with alkaline phosphatase. Incubation of the enzyme extracts under phosphorylating conditions produced only a slight further increase in the activity of enzyme from the treated slices, so that this

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Table 1. Effects of alkaline phosphatase and phosphorylating conditions on the increases in tryptophan hydroxylase activity induced by various treatments to rat brainstem slices

	Tryptophan hydroxylase activity (pmoles 5-HTP/mg protein/min)		
		Slice treatment	
Additions to assay	None	K^+ (66 mM)	A23187 (50 μM)
None	192 ± 5	364 ± 12†	363 ± 9†
Alkaline phosphatase*	191 ± 3	192 ± 3	190 ± 3
	(4)	(4)	(4)
None	183 ± 3	316 ± 9†	309 ± 4†
Phosphorylating conditions‡,§	346 ± 7§	347 ± 7‖	338 ± 9§
	(5)	(5)	(5)
		Slice treatment	
	None	IBMX (1 mM)	Guanidine (5 mM)
None	196 ± 6	352 ± 18†	350 ± 20†
Alkaline phosphatase*	196 ± 6	198 ± 5§	191 ± 3§
	(4)	(4)	(4)
None	182 ± 1	329 ± 8†	307 ± 7†
Phosphorylating conditions‡	357 ± 1 0§	330 ± 18	349 ± 11‖
	(4)	(4)	(4)

Values are means ± SEM; the number of individual brainstem slice preparations is given in parentheses.

* For the alkaline phosphatase treatment, 1 unit of alkaline phosphatase (Type IIIS, *Escherichia coli*, Sigma Chemical Co.) was incubated with the enzyme supernatant for 5 min at 37° prior to the addition of the assay reactants.

† Significantly different than activity of enzyme from untreated slices ($P < 0.01$).

‡ Phosphorylating conditions: 0.5 mM ATP, 5 mM $MgCl_2$, 0.05 $CaCl_2$.

§,‖ Significantly different than activity of enzyme from the same slice treatment group, but without additions to the assay: § $P < 0.01$, and ‖ $P < 0.05$.

now equalled that of control extracts in the presence of phosphorylating conditions (Table 1), but never exceeded it. The nonadditivity of the increases in enzyme activity induced in the slices and the increase obtained under phosphorylating conditions combined with the evidence of reversibility by alkaline phosphatase suggest a role for a phosphorylation reaction in mediating the increases in enzyme activity induced by the different treatments to the brainstem slices. None of these data, however, allows a distinction to be made between direct phosphorylation of the enzyme itself or of an activator molecule. The fact that control enzyme activity was unchanged by alkaline phosphatase treatment suggests that the control enzyme is not in an activated state. However, Lysz and Sze did find that the basal activity of supernatant extracts is lowered by acid phosphatase [9], raising the possibility that other phosphorylation sites on tryptophan hydroxylase (or an activator molecule) may contribute to basal enzyme activity. However, this inactivation could also have been due to a nonspecific loss of enzyme activity. The precise role of phosphorylation in the *in vivo* or *in situ* regulation of tryptophan hydroxylase activity cannot be resolved until phosphorylation sites can be studied by peptide mapping of ^{32}P-labeled, immunoprecipitated enzyme.

A role for calmodulin in the activation process is supported by the results in Table 2 showing that W-7 [10, 11], a calmodulin antagonist, completely blocked the increase in enzyme activity induced by the four different treatments to the slice preparations at a concentration of 100 μM (Table 2). The site of action of W-7 is an intracellular one and does not involve membrane interactions [10, 11]. Within the cell, however, W-7 not only blocks calmodulin-dependent processes, but also inhibits the calcium-sensitive, diacylglycerol-activated protein kinase, protein kinase C, in about the same concentrations that it binds to calmodulin [12]. Therefore, to test whether protein kinase C contributes to the activation of tryptophan hydroxylase, the slices of brainstem were incubated with polymixin B, an inhibitor of kinase C which readily enters cells [12, 13]. In concentrations of 100 μM, which are sufficient to produce complete inhibition of protein kinase C [12, 13], polymixin B had no effect on the increase in tryptophan hydroxylase activity induced by ionophore A23187, IBMX or guanidine. It did, however, block the increase obtained with potassium depolarization. This block could result from nonspecific, local anaesthetic effects of the drug in the neuronal membrane which would then interfere with the depolarization-induced entry of calcium that is required for activation of tryptophan hydroxylase. Indeed, when polymixin B was preincubated with the tissue slices to permit interaction with protein kinase C, but omitted from the depolarizing medium, then no blocking effect was observed on the activation of tryptophan hydroxylase (Table 2). In contrast to W-7, polymixin B did not affect the increase in enzyme activity obtained by incubation of extracts under phosphorylating conditions, whereas W-7 completely inhibited the activation of the enzyme under these conditions (data not shown). Thus, the inhibitory effects of W-7 reported here appear likely to be due to its interaction with calmodulin and provide support for involvement of calmodulin in the activation of tryptophan hydroxylase in the slice preparations.

In summary, the activation of tryptophan hydroxylase induced by treatments to slices of rat brainstem was revers-

Table 2. Effects of W-7, a calmodulin antagonist, and polymixin B, a protein kinase C inhibitor, on the increase in tryptophan hydroxylase activity induced by treatments to slices of brainstem

Additions to slice incubation medium	Tryptophan hydroxylase activity (pmoles 5-HTP/mg protein/min) Slice treatment None	K^+ (66 mM)	A23187 (50 μM)	IBMX (1 mM)	Guanidine (5 mM)
W-7* concn. (μM)					
0	201 ± 3	417 ± 14	395 ± 11	394 ± 12	373 ± 10
50		309 ± 5	296 + 9	296 ± 6	292 ± 5
100	200 ± 2	207 ± 6	195 ± 5	204 ± 2	190 ± 4
Polymixin B (μM)					
0	191 ± 2	377 ± 9	377 ± 10	378 ± 6	363 ± 6
100	191 ± 2	193 ± 1†	362 ± 11	376 ± 6	370 ± 10
		373 ± 8‡			

W-7 and polymixin B were preincubated with the slices of brainstem for 10 min prior to addition of slices to treatment medium containing W-7 or polymixin B. Brainstem slice preparations exposed to IBMX or guanidine were incubated in calcium-free control medium with 0.1 mM EGTA. Since the activity of tryptophan hydroxylase from slices incubated in unmodified control or calcium-free control slice incubation medium with or without added W-7 or polymixin B did not differ, these data were pooled (N = 16 and 12 respectively) for W-7 and polymixin B experiments. Values are means ± SEM; the minimum number of brainstem slice preparations for each treatment was four.

* Enzyme activity of all groups of treated slices containing 0 or 50 μM W-7 was significantly different from control slices (0 or 100 μM W-7) at the level of $P < 0.01$. At 100 μM W-7, differences were abolished. W-7 (50 or 100 μM) significantly lowered activity of enzyme from treated slices ($P < 0.01$ compared with corresponding treatment group without W-7). The significance of the difference in activity between enzyme exposed to 50, compared with 100 μM W-7, for a given treatment was $P < 0.01$.

† $P < 0.01$ compared to activity of enzyme from depolarized slices in the absence of polymixin B.

‡ Polymixin B was preincubated with slices in control medium but not added to the depolarizing medium.

ible by alkaline phosphatase, nonadditive with the activation induced by phosphorylating conditions and inhibited by W-7, a calmodulin antagonist. The most parsimonious explanation for these data is that this enzyme activation involves both calmodulin and a phosphorylating system presumably the calcium–calmodulin sensitive protein kinase, or calmodulin kinase II that has been found to activate the enzyme *in vitro*.

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Department of Physiology MARGARET C. BOADLE-BIBER*
Medical College of Virginia TAM-HAO PHAN
Virginia Commonwealth University
Richmond, VA 23298, U.S.A.

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* Address for correspondence: Dr. Margaret C. Boadle-Biber, Department of Physiology, Medical College of Virginia, Box 551, MCV Station, Richmond, VA 23298-0001.